Wnt Signaling Influences the Development of Murine Epidermal Langerhans Cells

Maria R. Becker[1], Yeon S. Choi[2], Sarah E. Millar[2] and Mark C. Udey[1]

Langerhans cells (LCs) are distinct dendritic cells (DCs) that populate stratified squamous epithelia. Despite extensive studies, our understanding of LC development is incomplete. Transforming growth factor β1 (TGFβ1) is required for LC development, but other epidermis-derived influences may also be important. Recently, EpCAM (CD326) has been identified as a cell surface protein discriminating LCs from Langerin$^+$ dermal DCs and other DCs in the skin. EpCAM is a known transcriptional target of the Wnt signaling pathway. We hypothesized that intraepidermal Wnt signaling might influence LC development. Addition of Wnt3A into cultures of bone-marrow-derived cells in combination with TGFβ1, GM-CSF, and M-CSF resulted in increased (33%; $P<0.05$) accumulation of EpCAM$^+$ DCs. In contrast, addition of the Wnt antagonist dickkopf-related protein 1 (Dkk1) decreased the number of EpCAM$^+$ DCs (21%; $P<0.05$). We used *K14-KRM1; K5-rtTA; tetO-Dkk1* triple-transgenic and *K5-rtTA; tetO-Dkk1* double-transgenic mice to test the *in vivo* relevance of our *in vitro* findings. Feeding doxycycline to nursing mothers induced expression of Dkk1 in the skin of transgenic pups, causing an obvious hair phenotype. Expression of Dkk1 reduced LC proliferation (40%; $P<0.01$) on P7, decreased LC densities (26%; $P<0.05$) on P14, and decreased EpCAM expression intensities on LCs as well (33%). In aggregate, these data suggest that Wnt signaling in skin influences LC development.

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INTRODUCTION

Epidermal Langerhans cells (LCs) represent a unique subset of dendritic cells (DCs) that populate stratified squamous epithelia. They have long been thought to have pivotal roles in initiating immunity by acquiring antigens that are encountered in skin, migrating to draining lymph nodes after activation, and stimulating antigen-specific T cells (Merad *et al.*, 2008). However, recent studies suggest that LCs do not function as essential antigen-presenting cells for anti-viral immune responses (Allan *et al.*, 2003; Aebischer *et al.*, 2005) or for contact hypersensitivity reactions (Bennett *et al.*, 2005, 2007; Kaplan *et al.*, 2005; Kissenpfennig *et al.*, 2005; Bursch *et al.*, 2007) in established murine models. Thus, despite extensive study, important aspects of LC physiology remain to be elucidated. Previous studies with transforming growth factor β1 (TGFβ1) (Borkowski *et al.*, 1996a) and M-CSF receptor (Ginhoux *et al.*, 2006) knockout mice demonstrated that TGFβ1 and M-CSF are essential for LC development. These cytokines likely act on local precursors (Bogunovic *et al.*, 2006) that proliferate *in situ* rather than circulating precursors (Merad *et al.*, 2008). However, additional epidermal-derived molecules that act only over short distances may also be relevant for LC development.

We previously determined that EpCAM (CD326) is expressed at high levels by murine LCs (Borkowski *et al.*, 1996b), and the ability of EpCAM expression to discriminate LCs from Langerin$^+$ dermal DCs and other DCs has been reported (Bursch *et al.*, 2007). EpCAM is a direct transcriptional target of the canonical Wnt-β-catenin signaling pathway (Yamashita *et al.*, 2007), and Wnt signaling is well known to be involved in epidermal development and homeostasis, and to participate in the development of hematopoietic cells (Korinek *et al.*, 1998; Saitoh *et al.*, 1998; Wodarz and Nusse, 1998; Clevers, 2006; Scheller *et al.*, 2006; Fleming *et al.*, 2008). Thus, we hypothesized that epidermis-derived Wnt proteins might regulate the development and/or homeostasis of EpCAM-expressing LCs in epidermis.

Binding of Wnt proteins to their receptors (frizzled proteins) and to members of the low-density lipoprotein receptor-related protein family that serve as essential coreceptors (LRP5 or LRP6) activates the canonical Wnt/β-catening signaling pathway. Pathway activation causes accumulation of β-catenin in the cytoplasm, translocation of β-catenin to the nucleus, formation of active transcription complexes of β-catenin and members of the LEF/TCF family of DNA-binding proteins, (Wodarz and Nusse, 1998;

[1]*Dermatology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA and* [2]*Department of Dermatology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA*

Correspondence: Mark C. Udey, Dermatology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Building 10, Room 12N238, Bethesda, Maryland 20892-1902, USA.
E-mail: udey@helix.nih.gov

Abbreviations: Cont, littermate controls without the doxycycline responsive transgene; DC, dendritic cell; Dkk1, dickkopf-related protein 1; DT, double transgenic; KRM1, kremen protein 1; LC, Langerhans cell; QRT-PCR, quantitative real-time RT-PCR; tetO, tetracycline operator; TT, triple transgenic

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Bejsovec, 2000) and, subsequently, transcription of genes that are regulated by Wnt-responsive elements. Wnt signaling is regulated via antagonists, including secreted Dickkopf-related protein 1 (Dkk1) (Glinka *et al.*, 1998; Niehrs, 1999, 2001). Dkk1 functions as an inhibitor of Wnt signaling by binding to kremen protein 1 (KRM1) (Mao *et al.*, 2002), a transmembrane high-affinity receptor, in conjunction with LPR5/6 (Bafico *et al.*, 2001; Semenov *et al.*, 2001), thereby promoting internalization of LRP5/6 and reduced responsiveness to Wnt (Wu *et al.*, 2000; Mao *et al.*, 2002).

Tissue-selective expression of Dkk1 in transgenic mice can be achieved with lineage-specific promoters and this approach has been used to modulate Wnt signaling *in vivo* (Huelsken *et al.*, 2001; Andl *et al.*, 2002; Chu *et al.*, 2004; Shu *et al.*, 2005; Ito *et al.*, 2007; Liu *et al.*, 2007; Osada *et al.*, 2010). In situations where constitutive inhibition of Wnt signaling is deleterious, mice with temporal as well as spatial regulation of Dkk1 expression can be utilized. *K5-rtTA; tetO-Dkk1* mice are double-transgenic (DT) animals that express a tetracycline reverse transactivator (rtTA) protein under the control of the keratin 5 promoter. The rtTA protein binds to tetracycline operator (tetO) elements in the presence of doxycycline, resulting in Dkk1 production in the skin of mice that are ingesting the antibiotic. These mice have been used previously to assess the involvement of Wnt signaling in mammary gland development (Chu *et al.*, 2004), wound healing in skin (Ito *et al.*, 2007), and thymus development (Osada *et al.*, 2010).

In this study, we also made use of triple-transgenic (TT) *K14-KRM1; K5-rtTA; tetO-Dkk1* mice that additionally include a Keratin14 promotor-driven KRM1 transgene, as KRM1 is a high-affinity Dkk1 receptor known to functionally cooperate with Dkk1 to inhibit Wnt signaling (Mao *et al.*, 2002). The combination of Dkk1 and KRM1 transgenes potentiates the inhibition of Wnt signaling in keratinocytes (Semenov *et al.*, 2001; Rothbacher and Lemaire, 2002). Although KRM1 single-transgenic mice do not display gross alterations in skin architecture or hair cycling, doxycycline-mediated Dkk1 induction in TT mice reveals an even more severe skin phenotype than that seen in DT *K5-rtTA; tetO-Dkk1* mice (Y.S. Choi and S.E. Millar, unpublished observations).

Studies of LC function have been constrained by the inability to routinely propagate LC-like cells *in vitro*. Although we previously described a methodology that allowed the generation of LC-like cells from fetal mouse skin (Jakob *et al.*, 1997), this primary culture system no longer supports expansion of cells of interest. Herein, we describe new conditions that allowed us to routinely propagate LC-like cells ($CD11c^+$ major histocompatibility complex (MHC) class II^+ $EpCAM^+$ DCs) from murine bone marrow. In the present studies, we assessed the ability of recombinant Wnt protein to promote the development of LC-like DCs *in vitro*, and the ability of the Wnt antagonist Dkk1 to inhibit LC development *in vivo* in *K5-rtTA; tetO-Dkk1* and *K14-KRM1; K5-rtTA; tetO-Dkk1* mice. Our results do not conclusively identify an essential role for Wnt signaling in LC development, but do suggest that Wnt signaling can influence LC proliferation, number, and phenotype.

RESULTS AND DISCUSSION

Generation of LC-like cells *in vitro*

In a series of preliminary experiments, we identified conditions that allowed optimal propagation of LC-like cells *in vitro*. The shape and size of the culture dishes used had a major impact on the development of $CD11c^+$ MHC class II^+ E-Cadherin$^+$ $EpCAM^+$ LC-like cells (Figure 1a). The largest numbers of total leukocytes and LC-like cells were obtained in 24-well plates. The time period after initiation of culture also influenced expression of various markers. After 72 hours, 10% of all cells expressed CD45, CD11c, MHC class II, E-Cadherin, EpCAM, and CD40. As expected, adding TGFβ1 into cultures prevented maturation of the LC-like cells, as manifested by expression of low levels of MHC class II and CD86 (Figure 1b). However, stimulation of LC-like cells with 100 ng ml^{-1} lipopolysaccharide for 22 hours in subcultures without TGFβ1 increased CD86 and MHC class II expression, indicating that these DCs were capable of maturation (data not shown). Langerin expression by cultured $EpCAM^+$ cells was low as compared with freshly isolated epidermal LCs. Quantitative real-time RT-PCR revealed an increase in Langerin mRNA expression by cultured LC-like cells over the first 72 hours. Accordingly, flow cytometry revealed a peak in intracellular Langerin protein expression after 96 hours (Figure 1c). The number of LC-like cells per well decreased after 120 hours.

In vitro effects of Wnt signaling modulators on LC-like cells

To investigate the involvement of Wnt signaling in LC development, we initially characterized the effects of Wnt protein and the Wnt antagonist Dkk1 on the development of murine LC-like DCs in C57BL/6 bone marrow cultures. Initial dose response studies revealed maximal effects of Wnt3A and Dkk1 at 100 and 1,000 ng ml^{-1}, respectively (data not shown). Addition of Wnt3A (100 ng ml^{-1}), which is known to activate the Wnt/β-catenin signaling pathway (Kishida *et al.*, 1999), into bone marrow cultures resulted in modest increases in the the number of LC-like DCs that were recovered after 72 hours (~33%, $P<0.05$, Figure 2a). In contrast, the potent Wnt inhibitor Dkk1 (1,000 ng ml^{-1}) decreased the number of LC-like cells accumulating in cultures that were not supplemented with Wnt3A protein (~21%, $P<0.05$, Figure 2a). Total leukocyte numbers, determined at 72 hours, did not change significantly in the presence of Wnt3A or Dkk1 (Figure 2b). These results indicate that Wnt3A has a modest selective effect on the development of LC-like cells *in vitro* and suggest that small amounts of endogenous Wnt proteins may be present and active in bone marrow cultures.

Influence of intraepidermal Wnt signaling on LCs *in vivo*

To assess the possible effects of Wnt signaling on LC development *in situ*, we initially characterized LCs in the epidermis of *K5-rtTA; tetO-Dkk1* DT mice (Supplementary Figure S1 online). Keratinocytes in these mice produce the Wnt inhibitor Dkk1 after exposure to doxycycline (Chu *et al.*, 2004). Dkk1 was induced in the skin of young mice by feeding doxycycline to nursing mothers beginning on

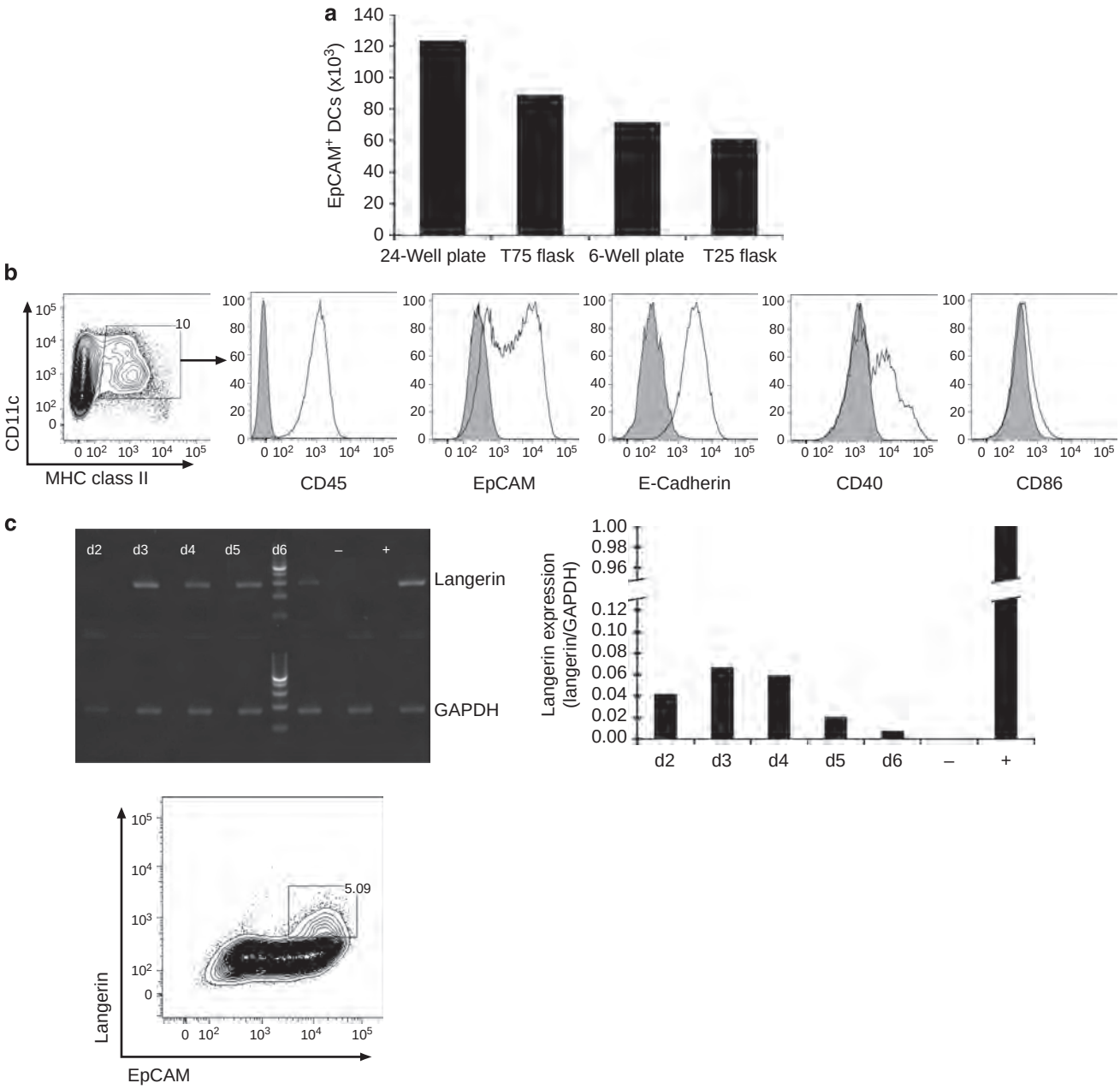

Figure 1. Generation of bone-marrow-derived Langerhans cell (LC)-like cells. (**a**) The shape and size of culture dishes had a major impact on the total number of EpCAM$^+$ dendritic cells (DCs) recovered. Data are expressed as total cell numbers of EpCAM$^+$ cells per culture dish per 10^6 input cells. (**b**) Phenotypes of LC-like cells after 72 hours as determined by flow cytometry. (**c**) Expression of Langerin by LC-like cells from day 2 until day 6 of culture as determined by RT-PCR. RNase-free water served as negative control (–), freshly isolated epidermal LCs served as positive control (+), and quantitative real-time RT-PCR data are expressed as fold expression in relation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and flow cytometry on day 4 of culture (gated on CD11c$^+$ major histocompatibility complex (MHC) class II$^+$ cells.

postnatal day 0 (P0). This approach avoids the limb and dental defects that would result from earlier exposure of developing mice to Dkk1 (Chu *et al.*, 2004). Owing to a lack of availability of the DT mice, we performed subsequent studies with *K14-KRM1; K5-rtTA; tetO-Dkk1* TT mice. In TT mice, the Wnt-inhibiting effect of Dkk1 is potentiated in keratinocytes by the additional expression of KRM1 in K14-expressing cells. The direct effects of Dkk1 on LCs or LC precursors are expected to be identical in DT and TT mice.

LC precursors enter murine skin soon after epidermal differentiation is completed and undergo a massive burst of proliferation between postnatal days 2 (P2) and 7 (P7), reaching ''adult'' numbers within the first 2 weeks after birth. (Kobayashi *et al.*, 1987; Chang-Rodriguez *et al.*, 2004, 2005; Tripp *et al.*, 2004; Chorro *et al.*, 2009; Elbe-Burger and Schuster, 2010). Thus, it was anticipated that an effect of Wnt inhibition by Dkk1 would be evident before P14 if Wnt proteins were involved in LC development.

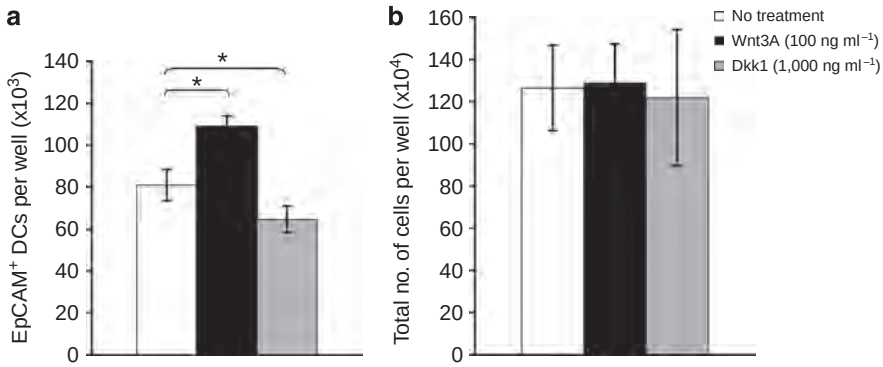

Figure 2. Effects of Wnt protein and a Wnt antagonist on the development of Langerhans cell (LC)-like dendritic cells (DCs) *in vitro*. (**a**) Aggregate results of effects of Wnt3A (100 ng ml^{-1}) and Wnt inhibitor dickkopf-related protein 1 (Dkk1) (1,000 ng ml^{-1}) on EpCAM$^+$ DC accumulation after 72 hours; untreated cells (no treatment) served as controls ($n=4$, *$P<0.05$). (**b**) Lack of effects of Wnt3A and Dkk1 on total leukocyte numbers in bone marrow cultures after 72 hours ($n=4$ experiments). Error bars represent the mean ± SEM.

Dkk1 induction resulted in an obvious body size and hair phenotype. DT and TT mice were smaller and had less terminal hair than their littermate controls. This confirms that administration of doxycycline to nursing mothers induced Dkk1 expression in the skin of pups with the appropriate genotype. Immunofluorescence staining of P7 TT epidermal sheets demonstrated that LCs in Dkk1-expressing epidermis were present and expressed MHC class II, Langerin, and EpCAM (Figure 3a). Enumeration of LCs in TT epidermal sheets on P7 did not reveal significant differences between TT mice and control animals. However, on P14, LC densities were ~26% lower in Dkk1-producing TT mice ($P<0.05$, Figure 3b), consistent with the lower LC densities (~21%, $P<0.05$) that had been previously observed in the P14 epidermis of DT mice (Supplementary Figure S1 online). Careful inspection revealed that LC morphology was also somewhat abnormal and anti-EpCAM immunofluorescence staining intensity was decreased in Dkk1-expressing mice. This impression was confirmed when laser-scanning cytometry measurements revealed that EpCAM staining intensities in the epidermis of Dkk1-expressing DT animals were decreased by ~33% as compared with controls, whereas MHC class II staining intensities were equivalent (Figure 3c).

Consistent with the decreased LC densities that were observed, Dkk1-producing TT mice also displayed a 40% decrease in LC proliferation on P7 as determined by quantifying Ki67 proliferation indices ($P<0.01$, Figure 4a and b). Interestingly, analysis of epidermal sheets revealed numerous MHC class II$^+$ cells at various stages of the mitosis (Figure 4c).

Multiple secreted proteins and differentiation signaling pathways may regulate proliferation of LC precursors in fetal/neonatal skin (Elbe-Burger and Schuster, 2010). Our *in vivo* experiments suggest that Wnt signaling regulates LC proliferation, but that it is not absolutely required for LC development in mice post-weaning. Our data also suggest that Wnt signaling influences murine LC phenotype and regulates EpCAM expression by LCs, as has been reported for other cells (Yamashita *et al.*, 2007; Munz *et al.*, 2009). It remains possible that Wnt signaling is essential for LC development at earlier stages of postnatal life than examined in the present study. As LCs survive for months to years in unperturbed epidermis, Wnt dependency might be very difficult to demonstrate after LC differentiation has been completed or even initiated. Additional studies regarding the factors and signaling pathways that regulate LC precursors in fetal mouse epidermis, and identification of culture conditions that allow routine propagation of LCs *in vitro* will be important for further characterization of this incompletely understood developmental process.

MATERIALS AND METHODS

Mice

Adult female C57BL/6 mice were obtained from the NCI-Fredrick Animal Production Program, Frederick, MD. *K5-rtTA; tetO-Dkk1* mice have been described previously (Chu *et al.*, 2004). FvB female *K5rtTA* Tg mice were mated to FvB male *tetO-Dkk1* Tg to generate *K5-rtTA; tetO-Dkk1* DT animals for study. These mice were additionally crossed to *K14-KRM1* mice to obtain TT animals. Littermates without the doxycycline responsive transgene were used as controls. Doxycycline was fed to nursing mothers beginning on postnatal day 0 (P0) to induce Dkk1 production in the epidermis of DT and TT animals. Mice were studied on P7 and P14, as indicated. All mice were bred and housed in a pathogen-free environment, and used in experiments in accordance with institutional guidelines. Mice were genotyped using tail clip DNA isolated via the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) used according to the manufacturer's protocol and PCR. PCR primers for rtTA (F: 5′AGCTGCTTAATGAGGTCGGA-3; R: 5′GCTTGTCGTAATAATGGCGG-3), Dkk1 (F: 5′-CCCGGATCCGCGTCCTTCGGAGATGATGG-3′; R: 5′-AATGGATCCTTTAGACTGTCGGTTTAGTGTCTC-3′), and KRM1 (F: 5′-CCGAGTGCAATAGTGTCTGC-3′; R: 5′-GGCTTGCTCGGTGATCACCTCCTC-3′) were used in conjunction with the following incubation conditions: 95 °C for 2 minutes, 75 °C for 95 seconds, and 35 repeats of a cycle at 95 °C for 30 seconds, 55 °C for 40 seconds and 72 °C for 2 minutes.

DC cultures

LC-like DCs were propagated as described previously (Inaba *et al.*, 2009), with some modifications. Briefly, femurs and tibias from

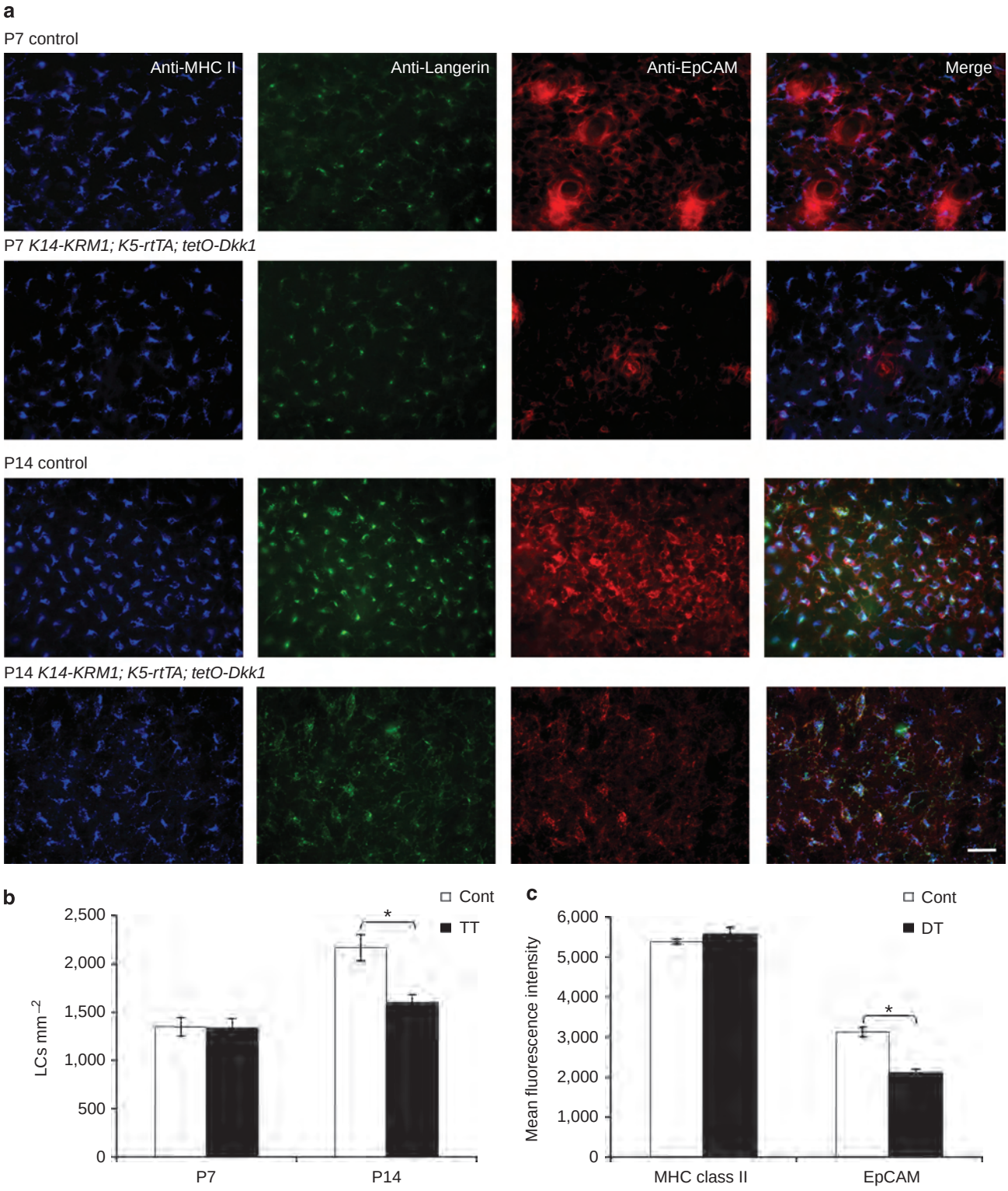

Figure 3. Langerhans cell (LC) densities and characteristics in mice that express a Wnt antagonist in epidermis. (**a**) Epidermal sheets on postnatal days 7 and 14 (P7, P14) from doxycycline-exposed littermate control (Cont) and *K14-KRM1; K5-rtTA; tetO-Dkk1* (triple transgenic (TT)) mice, stained with anti-EpCAM, anti-Langerin, anti-major histocompatibility complex (MHC) class II mAb, and visualized via immunofluorescence microscopy (bar = 25 μm). (**b**) LC densities from TT and Cont mice on P7 and P14 (three random fields per mouse, $n = 5$ mice per group, $^*P < 0.05$). (**c**) Mean fluorescence intensities of MHC class II and EpCAM expression in epidermal sheets from *K5-rtTA; tetO-Dkk1* (double transgenic (DT)) and Cont mice as determined with a laser scanning cytometer (see Materials and Methods, three random fields per mouse, $n = 2$ mice per group, $^*P < 0.05$). Error bars represent the mean ± SEM.

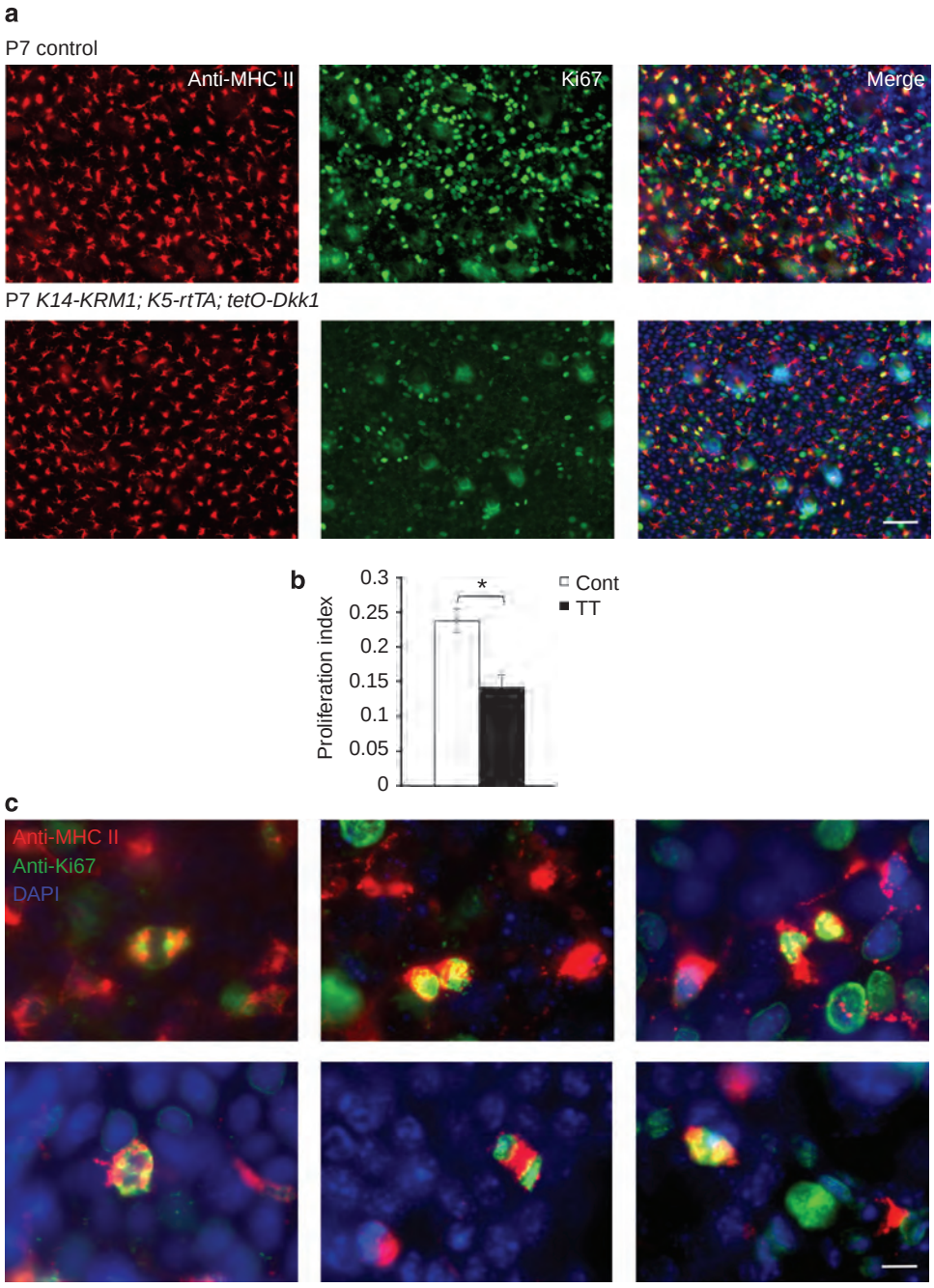

Figure 4. Langerhans cell (LC) proliferation in mice that express a Wnt antagonist in epidermis. (**a**) Epidermal sheets were prepared on postnatal day 7 (P7) from doxycycline-exposed littermate control (Cont) and *K14-KRM1; K5-rtTA; tetO-Dkk1* (triple transgenic (TT)) mice, stained with anti-major histocompatibility complex (MHC) class II mAb and anti-Ki67 polyclonal antibody, and visualized via immunofluorescence microscopy (bar = 50 μm). (**b**) LC proliferation indices as determined by dividing MHC class II^+ $Ki67^+$ cells by MHC class II^+ cells (three random fields per mouse, $n = 5$, $^*P < 0.01$). Error bars represent the mean ± SEM. (**c**) Various stages of mitosis in MHC class II^+ cells as depicted by Ki67 staining (bar = 15 μm).

C57BL/6 mice were flushed, and recovered cells were counted and resuspended at 10^6 cells ml^{-1} in MEM-α medium (Invitrogen, Carlsbad, CA), containing 10% heat-inactivated fetal bovine serum (HyClone Thermo Scientific, Waltham, MA), 2 mM glutamine, 0.1 mM non-essential amino acids, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1% PenStrep (all Invitrogen), 50 μM 2-mercaptoethanol (Sigma, St Louis, MO), and the recombinant cytokines human TGFβ1, murine GM-CSF, and murine M-CSF (all PeproTech, Rocky Hill, NJ) at concentrations of 10 ng ml^{-1} each. Recombinant murine Wnt3A and Dkk1 were purchased from R&D Systems (Minneapolis, MN). Wnt3A was provided in lyophilized form from phosphate-buffered saline, 0.1 mM EDTA, and 0.5% (w/v) CHAPS, pH 6.8 with BSA as a carrier protein. Diluent controls were utilized as indicated.

Antibodies

Purified rat IgG2aκ anti-mouse Langerin mAb (clone L31) and the corresponding isotype control were purchased from eBioscience (San Diego, CA) and labeled with Alexa Fluor 488 or 647 using mAb labeling kits (Invitrogen). Polyclonal rabbit anti-Ki67 (Abcam, Cambridge, MA) was used in combination with a donkey anti-rabbit Alexa 488-labeled secondary Ab (Invitrogen). Additional directly labeled mAb and their isotype controls (BD Biosciences, San Jose, CA unless otherwise indicated) were used for immunofluorescence microscopy and flow cytometry to detect the following: EpCAM (Alexa Fluor 488 or 647-G8.8, BioLegend, San Diego, CA), CD11c (APC-HL3), and MHC class II (FITC-M5/114.15.2). Rat anti-mouse CD16/32 (2.4G2) and rat IgG2aκ were routinely used for blocking (2.5 μg ml^{-1}) before staining; for Ki67 staining 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) was added into the blocking buffer.

Flow cytometry

Data were collected with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR). Non-viable cells were excluded after 7-AAD (BD Biosciences) staining, unless cells had been fixed and permeabilized (Cytofix/Cytoperm Kit, BD Biosciences) for langerin detection before analysis.

Assessment of Langerin mRNA expression by LC-like cells and LCs

Cultured LC-like cells were enriched for EpCAM$^+$ cells by incubation with Alexa Fluor 647-labeled anti-mouse EpCAM mAb (G8.8 clone) and positive selection using anti-Alexa Fluor 647 magnetic beads and the MACS Separation Unit (Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA from the EpCAM$^+$ cells was extracted using TRIzol Reagent (Invitrogen), purified via the RNeasy Mini Kit (Qiagen), and used to prepare complementary DNA with SuperScript III First-Strand Synthesis SuperMix (Invitrogen) in accordance with the manufacturer's protocols. As a positive control, freshly isolated LCs were prepared from epidermal cell suspensions (Tang *et al.*, 1993) using Lympholyte M (Cedarlane Laboratories Limited, Burlington, NC) density gradients. Interface cells were further enriched for EpCAM$^+$ cells using magnetic beads as mentioned above. Flow cytometry of the positive selected cell fraction ensured an enrichment of 95% EpCAM$^+$ cells (data not shown). RNase-free water served as the negative control.

Semi-quantitative PCR was performed using Platinum PCR SuperMix (Invitrogen) as well as primers for Langerin (5′-ACG CACCCCAAAGACCTGGTACAG-3′, 5′-AGACACCC TGATATTGG CACAGTG-3′) and glyceraldehyde-3-phosphate dehydrogenase, and cycling conditions of 95 °C for 5 minutes, 30 repeats of cycles at 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 minute, and a final extension at 72 °C for 7 minutes.

Quantitative PCR was performed using Maxima SYBR Green qPCR Master Mix (Fermentas Thermo Scientific, Glen Burnie, MD), primers for Langerin and glyceraldehyde-3-phosphate dehydrogenase, and cycling conditions of 95 °C for 10 minutes, 40 repeats of cycles at 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 minute, and a final extension at 65 °C for 7 minutes.

Preparation of epidermal sheets

Ears were split into dorsal and ventral halves, cartilage and subcutaneous tissue were removed, and skin was floated on 3.8% ammonium thiocyanate (Sigma) in phosphate-buffered saline for 20 minutes at 37 °C. The epidermis was separated from dermis and fixed in acetone at 20 °C for 15 minutes before rehydration in phosphate-buffered saline.

Immunofluorescence microscopy

Rehydrated epidermal sheets were incubated in 3% dry milk-phosphate-buffered saline (Bio-Rad Laboratories, Hercules, CA) including 5 μg ml^{-1} rat anti-CD16/32 mAb (BD Biosciences) for 1 hour at room temperature to minimize nonspecific staining before incubation with fluorochrome-labeled mAb for 1 hour at room temperature or overnight at 4 °C. For Ki67 staining, 5% donkey serum was added into the blocking buffer. Labeled cells were visualized using a Zeiss AxioImager A1 Imunofluorescence Microscope (Carl Zeiss, Oberkochen, Germany). Intensities of digital images in experimental and control specimens were adjusted within the linear range with Zeiss Axiovison software (Carl Zeiss). LC densities and Ki67 proliferation indices were determined by counting at least three random fields per animal at ×200 final magnification. The latter was obtained by dividing the number of Ki67/MHC class II double-positive cells by MHC class II-positive cells in each epidermal sheet. Mean fluorescence intensities corresponding to expression of MHC class II and EpCAM in microscopic fields in epidermal sheets were determined using a Compucyte Laser Scanning Cytometer and the iCYS 3.4 software (CompuCyte Corporation, Westwood, MA).

Statistics

P values were calculated with Microsoft Excel 2008 for Mac using the Student's *t*-test ($P<0.05$ was considered to be statistically significant). Error bars represent the mean ± SEM, *n* as indicated.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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